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(54) **Recombinant production of carotenoids, particularly astaxanthin**

(57) The present invention is directed to a process for producing carotenoids comprising cultivating a recombinant organism having a gene for one or more active oxygen species-quenching factor(s) that is substantially disrupted with a disruption cassette specific to the

gene, and recovering carotenoids from the culture, as well as to genetic materials useful in the said process, such as a recombinant organism producible of carotenoids, a disruption cassette, a recombinant DNA sequence, a recombinant DNA fragment, and a polynucleotide.

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Description

[0001] The present invention relates to recombinant production of carotenoids, particularly astaxanthin, and biological materials useful therefor.

[0002] Astaxanthin is known to be distributed in a wide variety of organisms such as animal (e.g. birds such as flamingo and scarlet ibis, and fish such as rainbow trout and salmon), algae and microorganisms. It is also recognized that astaxanthin has a strong antioxidation property against active oxygen as well as most of carotenoids, which is expected to apply to pharmaceutical usage to protect living cells against some diseases such as a cancer. Moreover, from a viewpoint of industrial application, a demand for astaxanthin as a coloring reagent is increasing especially in the industry of farmed fish, such as salmon, because astaxanthin imparts distinctive orange-red coloration to the animals and contributes to consumer appeal in the marketplace.

[0003] *Phaffia rhodozyma* is known as a carotenogenic yeast strain which produces astaxanthin specifically. Different from the other carotenogenic yeast, *Rhodotorula* species, *Phaffia rhodozyma* can ferment some sugars such as D-glucose. This is an important feature from a viewpoint of industrial application. In a recent taxonomic study, a sexual cycle of *P. rhodozyma* was revealed and its teleomorphic state was designated under the name of *Xanthophyllomyces dendrorhous* (W.I. Golubev; *Yeast* 11, 101 - 110, 1995). Some strain improvement studies to obtain hyper producers of astaxanthin from *P. rhodozyma* have been conducted, but such efforts have been restricted to employ the method of conventional mutagenesis and protoplast fusion in this decade. Recently, Wery *et al.* developed a host vector system using *P. rhodozyma* in which a non-replicable plasmid was used to be integrated onto the genome of *P. rhodozyma* at the locus of ribosomal DNA in multicopies (Wery *et al.*, *Gene*, 184, 89-97, 1997). And Verdoes *et al.* reported more improved vectors to obtain a transformant of *P. rhodozyma* as well as its three carotenogenic genes which code for the enzymes that catalyze the reactions from geranylgeranyl pyrophosphate to betacarotene (International patent WO97/23633). The importance of genetic engineering method on the strain improvement study of *P. rhodozyma* will increase in near future to break through the reached productivity by the conventional methods.

[0004] As described above, astaxanthin has an antioxidant property and this feature seems to have an important role *in vivo* for a protection against active oxygen species such as O_2^- , H_2O_2 and OH^\cdot , which are continuously generated in living cells. An *et al.* obtained hyperproducer of astaxanthin from *P. rhodozyma* by selection of antimycin-sensitive strain after a conventional chemical mutagenesis (An, G-H. *et al.*, *Appl. Env. Microbiol.*, 55 (1), 116-124, 1989). Antimycin is known to be an inhibitor of respiratory chain between cytochrome b and C_1 (Lucchini, G. *et al.*, *Mol. Gen. Genet.*, 177, 139-, 1979) and enhanced pigmentation in such an antimycin-sensitive mutants. Active oxygen species produced due to a blockade of the primary respiratory chain at the bc_1 complex stimulated carotenoid formation (An, G-H *et al.*, *Appl. Env. Microbiol.*, 55, 116-124, 1989). Indeed, addition of an O_2^- generator, duroquinone, to the growth medium increased total carotenoid content (main carotenoid is astaxanthin) as well as the relative amounts of xanthophylls present in *P. rhodozyma*, while the active oxygen species-quenching factor mannitol reversed this effect (Schroeder, W. A. *et al.*, *J. Gen. Microbiol.*, 139, 907-912, 1993). These results prompted such authors to speculate an antioxidant property of astaxanthin in *P. rhodozyma*. In fact, astaxanthin production is stimulated in post-exponential growth phase when respiration activity is fully induced. Moreover, the addition of respiratory substrate such as ethanol to the medium enhanced the astaxanthin production in *P. rhodozyma* (Gu, W-L. *et al.*, *J. Ind. Microbiol. Biotechnol.*, 19, 114-117, 1997). Although Schroeder *et al.* tried to determine a relationship of activities of superoxide dismutase (SOD) and catalase which act as native active oxygen species-quenching factors in *P. rhodozyma* to the productivity of astaxanthin by comparing a difference between parent strain and antimycin-sensitive hyperproducer of astaxanthin, direct correlation of *in vitro* activity could not be observed.

[0005] In accordance with the present invention the genes and the enzymes for active oxygen species-quenching factor(s), such as SOD and catalase which are biological materials useful in the improvement of the carotenoid, particularly astaxanthin, production process are provided. This invention involves cloning and determination of the genes which code for mitochondrial and cytoplasmic SODs and catalase. This invention also involves the enzymatic characterization as a result of the disruption of the genes in *P. rhodozyma*. Their disruption effects on the carotenogenesis can be confirmed by the cultivation of such transformants in an appropriate medium under an appropriate cultivation condition. More particularly, the present invention provides a process for producing carotenoids which comprises cultivating a recombinant organism whose gene for one or more active oxygen species-quenching factor(s) is substantially disrupted with the aid of a disruption cassette specific to the said gene, and recovering carotenoids from the culture. The host organism of the said recombinant organism may belong to the kingdom of *Monera*, *Protista* or *Fungi*. More preferable host organism of the said recombinant organism may belong to the genus *Erwinia*, *Rhodobacter*, *Myxococcus*, *Flavobacter*, *Paracoccus*, *Synechococcus*, *Synechocystis*, *Agrobacterium*, *Streptomyces*, *Haematococcus*, *Dunaliella*, *Phaffia*, *Xanthophyllomyces*, *Neurospora*, *Rhodotorula*, *Blakeslea*, or *Phycomyces*. Most preferably, the said host organism is a strain of *P. rhodozyma*.

[0006] The active oxygen species-quenching factor(s) is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD) and/or catalase.

[0007] The present invention also provides a recombinant organism producible of carotenoids, which is characterized in that the gene for at least one active oxygen species-quenching factor is substantially disrupted with the aid of introduction of a disruption cassette specific to the said gene. The active oxygen species-quenching factor(s) to be disrupted is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD) and/or catalase.

[0008] The present invention further provides a disruption cassette which can be used to prepare the said recombinant organism of the present invention for disrupting a gene coding for an active oxygen species-quenching factor effective in an organism which is producible of carotenoids, which comprises a partial nucleotide sequence substantially identical to a part of the DNA sequence coding for an active oxygen species-quenching factor and a selectable marker gene. For the construction of the disruption cassette, the target organism may belong to the kingdom of *Monera*, *Protista* or *Fungi*, more preferably to the genus *Erwinia*, *Rhodobacter*, *Myxococcus*, *Flavobacter*, *Paracoccus*, *Synechococcus*, *Synechocystis*, *Agrobacterium*, *Streptomyces*, *Haematococcus*, *Dunaliella*, *Phaffia*, *Wanophyllum*, *Neurospora*, *Rhodotorula*, *Blakeslea*, or *Phycomyces*. The active oxygen species-quenching factor(s) to be disrupted is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD) and/or catalase.

[0009] As used herein, a polynucleotide or polypeptide sequence (A) is said to be substantially identical to another sequence (B) if sequence A is at least 75% identical, preferably 85% identical, such as at least 95% identical to sequence B.

[0010] Further, the present invention provides a recombinant DNA sequence coding for an active oxygen species-quenching factor effective in an organism producible of carotenoids. The DNA sequence may be originated from an organism belonging to the kingdom of *Monera*, *Protista* or *Fungi*, more preferably to the genus *Erwinia*, *Rhodobacter*, *Myxococcus*, *Flavobacter*, *Paracoccus*, *Synechococcus*, *Synechocystis*, *Agrobacterium*, *Streptomyces*, *Haematococcus*, *Dunaliella*, *Phaffia*, *Xanthophyllum*, *Neurospora*, *Rhodotorula*, *Blakeslea*, or *Phycomyces*. Particularly preferred organism is *P. rhodozyma*. The active oxygen species-quenching factor coded by the recombinant DNA sequence may be mitochondrial superoxide dismutase, cytoplasmic superoxide dismutase and/or catalase.

[0011] A recombinant DNA sequence coding for mitochondrial superoxide dismutase can be identified by SEQ ID NO: 1 or 4, or it may have high homology to the sequence of SEQ ID NO: 1 or 4 enough to recombine with either of the sequences of SEQ ID NOs: 1 and 4. A recombinant DNA sequence coding for cytoplasmic superoxide dismutase can be identified by SEQ ID NO: 2 or 6, or it may have high homology to the sequence of SEQ ID NO: 2 or 6 enough to recombine with either of the sequences of SEQ ID NOs: 2 and 6. Also a recombinant DNA sequence coding for catalase can be identified by SEQ ID NO: 3 or 8, or it may have high homology to the sequence of SEQ ID NO: 3 or 8 enough to recombine with either of the sequences of SEQ ID NOs: 3 and 8.

[0012] In the present invention, any combination of the following hybridization and wash conditions may be used, as appropriate, to identify homologous polynucleotide sequences:

High Stringency Hybridization:

[0013]

6X SSC
0.5% SDS
100 ug/ml denatured salmon sperm DNA
50% formamide
Incubate overnight with gentle rocking at 42°C overnight.

High Stringency Wash:

[0014] 1 wash in 2X SSC, 0.5% SDS at Room Temperature for 15 minutes, followed by another wash in 0.1X SSC, 0.5% SDS at Room Temperature for 15 minutes.

Low Stringency Hybridization:

[0015]

6X SSC
0.5% SDS
100 ug/ml denatured salmon sperm DNA
50% formamide
Incubate overnight with gentle rocking at 37°C overnight.

Low Stringency Wash:

[0016] 1 wash in 0.1X SSC, 0.5% SDS at Room Temperature for 15 minutes.

[0017] Moderately stringent conditions may be obtained by varying the temperature at which the hybridization reaction occurs and/or the wash conditions as set forth above.

[0018] Thus, as used herein, a sequence (A) is said to have "high homology" to another sequence (B) if sequence A hybridizes to sequence B under high stringency conditions (*i.e.*, high stringency hybridization and wash conditions as defined above), and if the polypeptide or polypeptide fragment encoded by sequence A has the same activity as the polypeptide encoded by B.

[0019] The present invention further provides a recombinant DNA fragment which comprises a coding region for a transit peptide upstream the coding region of an objective protein which may be mitochondrial superoxide dismutase. The expression of this recombinant DNA fragment enables to locate the objective protein in mitochondria. Thus the present invention also provides a method for locating an objective protein in mitochondria which comprises expressing the recombinant DNA fragment comprising a coding region for a transit peptide upstream the coding region of an objective protein in an appropriate recombinant host organism.

[0020] As mentioned above, the present invention discloses the nucleotide sequences of active oxygen species-quenching factors, such as mitochondrial superoxide dismutase, cytoplasmic superoxide dismutase and catalase. These polynucleotides are provided for use as probe or primer for cloning gene for the active oxygen species-quenching factor(s) effective in another organism producible of carotenoids on the basis of the homology of the genes.

[0021] FIG. 1 shows the activity staining for superoxide dismutase after native polyacrylamide gel electrophoresis by using cell-free extracts obtained from ATCC 96594 and its *SOD* mutants. Lane 1, *P. rhodozyma* ATCC 96594; Lane 2 *P. rhodozyma* ATCC 96594 :: pSOD/G717 (*SOD1* disruptant); Lane 3 *P. rhodozyma* ATCC 96594 :: pSOD/G828 (*SOD2* disruptant); Lane 4 *P. rhodozyma* ATCC 96594

[0022] As it is briefly explained above, the object of the present invention is to provide a novel process for producing carotenoids biologically. The novel process comprises cultivating a recombinant organism whose gene for one or more active oxygen species-quenching factor(s) is substantially disrupted with the aid of a disruption cassette specific to the said gene, and recovering carotenoids from the culture.

[0023] For the purpose of the present invention, there is also provided a recombinant DNA sequence which contains an open reading frame coding for active oxygen species-quenching factor(s), which may be an enzyme, such as mitochondrial *SOD* or cytoplasmic *SOD*. The recombinant DNA sequence may contain a partial fragment encoding catalase gene. These sequences are useful to construct the said disruption cassette, as they are able to recombine with the native gene(s) for the said enzymes so as to specifically disrupt the gene(s).

[0024] The said recombinant DNA sequence may be the one which can be derived from an organism belonging to the kingdom of *Monera*, *Protista* or *Fungi*, more preferably to the genus *Erwinia*, *Rhodobacter*, *Myxococcus*, *Flavobacter*, *Paracoccus*, *Synechococcus*, *Synechocystis*, *Agrobacterium*, *Streptomyces*, *Haematococcus*, *Dunaliella*, *Phaffia*, *Xanthophyllomyces*, *Neurospora*, *Rhodotorula*, *Blakeslea*, or *Phycomyces*. Particularly preferred organism is *P. rhodozyma*. The active oxygen species-quenching factor coded by the recombinant DNA sequence may be mitochondrial *SOD*, cytoplasmic *SOD* and/or catalase. A specific example of the recombinant DNA sequence is derived from a gene of *Phaffia rhodozyma* and is selected from (i) a DNA sequence represented in SEQ ID NO: 1 or 2; (ii) those cDNAs identified by SEQ ID NO: 4 or 6; (iii) an isocoding or an allelic variant for the DNA sequence represented in SEQ ID NO: 1, 2, 4 or 6; and (iv) a derivative of a DNA sequence represented in SEQ ID NO: 1, 2, 4 or 6 with addition, insertion, deletion and/or substitution of one or more nucleotide(s), and coding for a polypeptide having the said enzyme activity. The said recombinant DNA sequence may also be characterized in that (a) it codes for the said enzyme having an amino acid sequence selected from the group consisting of those described in SEQ ID NOs: 5 and 7, or (b) it codes for a variant of the said enzyme selected from (i) an allelic variant, and (ii) an enzyme having one or more amino acid addition, insertion, deletion and/or substitution and having the stated enzyme activity.

[0025] As used herein, an "allelic variant" means the variant that has at least one mutation in either one of two alleles in the diploid organism such as *Phaffia rhodozyma*, *Xanthophyllomyces dendrorhous* and the like. Both alleles of a given gene are concerned with the same trait or characteristic, but the product or function coded for by a particular allele differs, qualitatively and/or quantitatively, from that coded for by other alleles of that gene.

[0026] Allelic variant can occur naturally or be generated artificially by means of chemical mutagenesis. A wild type allele is one which codes for a particular phenotypic characteristic found in the wild type strain of a given organism.

[0027] As used herein, an "isocoding variant" means the variant in which the nucleotide sequence of a given gene differs from the sequence from the wild type gene although its translated product (*i.e.* amino acid sequence) is identical with that from the wild type protein. This is caused by degeneracy of genetic code and by the difference of codon usage which is not identical among various organisms.

[0028] As used herein, a "derivative of a DNA sequence" is a DNA sequence that encodes a polypeptide having the activity of the corresponding SEQ ID NO but which differs from that DNA sequence by from 1-20, preferably 1-10, such

as 1-5 nucleotide additions, insertions, deletions, and/or substitutions.

[0029] Particularly specified recombinant DNA sequence mentioned above may be that which can be derived from a gene of *Phaffia rhodozyma* and is selected from (i) a DNA sequence represented in SEQ ID NO: 3; (ii) cDNA identified by SEQ ID NO: 8; (iii) an isocoding or an allelic variant for the DNA sequence represented in SEQ ID NO: 3 or 8; and (iv) a derivative of a DNA sequence represented in SEQ ID NO: 3 or 8 with addition, insertion, deletion and/or substitution of one or more nucleotide(s), and coding for a polypeptide having the said enzyme activity. The said recombinant DNA sequence may also be characterized in that (a) it codes for the said enzyme having a partial amino acid sequence selected from the group consisting of those described in SEQ ID NO: 9, or (b) it codes for a variant of the said enzyme selected from (i) an allelic variant, and (ii) an enzyme having one or more amino acid addition, insertion, deletion and/or substitution and having the stated enzyme activity. Such a recombinant DNA sequence may preferably be in the form of a vector.

[0030] The present invention also provides the use of the said recombinant DNA sequence to transform a host organism so as to obtain an organism whose gene for at least one active oxygen species-quenching factor is substantially disrupted with the aid of introduction of the said disruption cassette specific to the said gene.

[0031] As used herein, a gene is "disrupted" or "substantially disrupted" if the activity of the polypeptide that it encodes is reduced relative to a non-disrupted gene. Preferably, the activity is reduced by 10%, preferably by at least 50% such as for example by at least 75%, more preferably by at least 90% to 100%.

[0032] A convenient form of the recombinant DNA sequence may be a vector. The recombinant organism obtained by use of the recombinant DNA is disrupted in its DNA sequence encoding mitochondrial SOD, cytoplasmic SOD, or catalase. The host organism transformed with the recombinant DNA is useful in the improvement of the production process of carotenoids, in particular astaxanthin. Thus the present invention also provides such a recombinant organism.

[0033] This biological production method of carotenoids can improve the productivity of carotenoids, in particular of astaxanthin. Thus the method for producing a carotenoid, characterized in that a recombinant organism described above is cultivated under conditions conducive to the production of the carotenoid is one of the aspect of the present invention. This method may be applied to the biological production of astaxanthin.

[0034] Many researchers pointed out that active oxygen species might stimulate carotenoid production in known carotenogenic organisms. Carotenoid biosynthesis in cyst cells of *Haematococcus pluvialis* is enhanced by environmental oxidative stress (Kobayashi et al., *Appl. Env. Microbiol.*, 59, 867-873, 1993). Carotenoid biosynthesis might be induced by active oxygen species and the accumulated carotenoids might function as a protective agent against oxidative stress damage in *Dunaliella bardawil* (Shaish et al., *Planta*, 190, 363-368, 1993). Although astaxanthin production in *P. rhodozyma* was studied *in vivo* under various cultivation conditions in which a generation of active oxygen species was altered, a correlation between active oxygen generated and carotenoids productivity was not clearly determined, probably because native active oxygen species-quenching factors were still present in such experiments and rescued the effects of active oxygen species on the carotenoids production to some extent (Schroeder, W. A. et al., *J. Gen. Microbiol.*, 139, 907-912, 1993).

[0035] In this invention, to exclude the possibility that existence of native active oxygen species-quenching factor in *P. rhodozyma* can quench the positive effect by active oxygen on astaxanthin production, such native active oxygen species-quenching factors as SOD and catalase were cloned from *P. rhodozyma* to disrupt their expression by constructing and introducing gene disruption plasmids. On the assumption that astaxanthin would play an antioxidant role in *P. rhodozyma*, inactivation of native active oxygen species-quenching factor may affect to carotenoids production probably because relatively increased active oxygen species *in vivo* due to absence of native active oxygen species-quenching factors would stimulate a production of astaxanthin as an alternative player to quench active oxygen species.

[0036] Active oxygen species have toxicity to living cells through the oxidative damage to the intercellular molecules such as proteins or nucleic acids. Recent studies revealed that aging is caused by oxidative damage by demonstrating a correlation between increased superoxide dismutase activity, increased life-span, and decreased oxidative damage in fruit flies and nematodes (Agarwal, S. et al., *Proc. Natl. Acad. Sci. U. S. A.*, 91, 12332-12335, 1994, Larsen, P. L. *Proc. Natl. Acad. Sci. U. S. A.*, 90, 8905-8909, 1993, Sohal, R. S. et al., *J. Biol. Chem.*, 270, 15671-15674, 1995). SOD and related antioxidant enzymes and their genes are studied well in procaryote and eukaryote.

[0037] Yeast, *S. cerevisiae*, like most eucaryotes, contains Cu/ZnSOD (product of the *SOD1* gene) in the cytosol and MnSOD (product of the *SOD2* gene) in the mitochondria. These enzymes catalyze the disproportionating of O_2 , producing O_2 and H_2O_2 . Together with small molecular antioxidants, such as glutathione and ascorbate; other antioxidant enzymes, such as catalases and peroxidases; and metal chelating proteins such as metallothionein, they allow aerobes to survive under O_2 , presumably by minimizing oxidative damage. The importance of cytoplasmic SOD was demonstrated by the high sensitivity to dioxygen shown by *S. cerevisiae* and *Escherichia coli* devoid of SOD. In both organisms, the loss of SOD activity is associated with slow growth in aerobic conditions, with higher mutation rates and with specific biosynthetic defects. (*sod1*⁻ yeast requires lysine and methionine for aerobic growth, whereas *sod*⁻ *E. coli* requires branched amino acids). In some cases these effects are known to be due to the inhibitory effect of

superoxide on iron sulfur cluster proteins (Gardner, P. R. *et al.*, *J. Biol. Chem.*, 266, 19328-19333, 1991, Kuo, C. F., *et al.*, *J. Biol. Chem.*, 262, 4724-4727, 1987). Mutants of *sod2* of *S. cerevisiae* are little affected when grown in air with glucose as the carbon source. However, they are highly sensitive to hyperoxia and grow poorly in normoxia in carbon sources that require respiration for their metabolism.

[0038] Because genes coding for SOD and catalase have been cloned from other species, corresponding genes from *P. rhodozyma* could be cloned with degenerate PCR method. At first, we cloned a partial gene fragment containing a portion of *SOD* gene and *CAT* gene by using above method. The said degenerate PCR is a method to clone a gene of interest which has high homology of amino acid sequence to the known enzyme from other species which has a same or similar function. Degenerate primer, which is used as a primer in degenerate PCR, was designed by a reverse translation of the amino acid sequence to corresponding nucleotides ("degenerated"). In such a degenerate primer, a mixed primer which consists any of A, C, G or T, or a primer containing inosine at an ambiguity code is generally used. In this invention, such the mixed primers were used for degenerate primers to clone above genes. PCR condition used is varied depending on primers and genes to clone as described hereinafter. In this invention, two species of *SOD* genes whose sequence was different from each other were cloned from the same PCR band in degenerate PCR and named as *SOD1* and *SOD2* genes.

[0039] An entire gene containing its coding region with its intron as well as its regulation region such as a promoter or terminator can be cloned from a chromosome by screening of genomic library which is constructed in phage vector or plasmid vector in an appropriate host, by using a partial DNA fragment obtained by degenerate PCR as described above as a probe after it was labeled. Generally, *E. coli* as a host strain and *E. coli* vector, a phage vector such as λ phage vector, or a plasmid vector such as pUC vector are often used in the construction of library and a following genetic manipulation such as a sequencing, a restriction digestion, a ligation and the like. In this invention, an *EcoR*I genomic library of *P. rhodozyma* was constructed in the derivatives of λ vector, λ ZAPII and λ DASHII depending on an insert size. An insert size, what length of insert must be cloned, was determined by the Southern blot hybridization for each gene before a construction of a library. In this invention, a DNA which was used for a probe was labeled with digoxigenin (DIG), a steroid hapten instead of conventional ^{32}P label, following the protocol which was prepared by the supplier (Boehringer-Mannheim (Mannheim, Germany)). A genomic library constructed from the chromosome of *P. rhodozyma* was screened by using a DIG-labeled DNA fragment which had a portion of a gene of interest as a probe. Hybridized plaques were picked up and used for further study. In the case of using λ DASHII (insert size was from 9 kb to 23 kb), prepared λ DNA was digested by the *EcoR*I, followed by the cloning of the *EcoR*I insert into a plasmid vector such as pUC19 or pBluescriptII SK+. When λ ZAPII was used in the construction of the genomic library, *in vivo* excision protocol was conveniently used for the succeeding step of the cloning into the plasmid vector by using a derivative of single stranded M13 phage, Ex assist phage (Stratagene, La Jolla, USA). A plasmid DNA thus obtained was examined for sequencing. In this invention, *SOD1* and *SOD2* genes were obtained from the λ ZAPII library independently from each other and catalase (*CAT*) gene was cloned from λ DASHII library.

[0040] In this invention, we used the automated fluorescent DNA sequencer, ALFred system (Pharmacia, Uppsala, Sweden) using an autocycle sequencing protocol in which the Taq DNA polymerase is employed in most cases of sequencing.

[0041] In this invention, the inventors determined the genomic sequence containing an open reading frame of *SOD1* gene or *SOD2* gene as well as its promoter and terminator sequence. From sequence analysis, it was found that *SOD1* codes for mitochondrial SOD, judged from a presence of transit peptide at its amino terminal end. On the contrary, *SOD2* does not have such a transit peptide sequence and this fact suggested that *SOD2* codes for cytoplasmic SOD. The inventors also determined a partial genomic sequence of an open reading frame for *CAT* gene.

[0042] Transit peptide is a signal sequence to transfer the nucleic gene products, which code on chromosome, and whose translated proteins function in mitochondria, to mitochondrial location of proteins, such as enzymes involved in TCA cycle. To express some proteins in mitochondria, addition of transit peptide at its amino terminal end is useful.

[0043] In this invention, disruption plasmids for *SOD1*, *SOD2* and *CAT* genes were constructed by ligating partial fragments of above genes, which do not contain the either end of genes, with drug resistant genes to suicidal vector, which cannot be autonomously replicated in *P. rhodozyma* due to lack of autonomous replication sequence. A drug resistant gene which encodes the enzyme that enables the host survive in the presence of a toxic antibiotic is often used for the selectable marker. G418 resistance gene harbored in pPR2T (Verdoes *et al.* (International patent publication, WO97/23633)) is an example of a drug resistance gene. Such a suicidal vector cannot replicate by itself and can be present only in an integrated form on the chromosome of the host as a result of a single-crossing recombination using the homologous sequence between a vector and a chromosome. In the case of recombination with a gene of interest, its genetic sequence cannot be reconstituted on the chromosome of the host strain due to lack of either end of the gene and as a consequence, the gene of interest could be disrupted in the recombinant strain thus obtained. Another example for disruption plasmid is a double crossing over type of plasmid. This type of disruption plasmid contains two different partial fragments of objective gene to be disrupted and selective marker gene such as a drug resistant gene is inserted between such two fragments. After the recombination between the chromosome of the re-

recipient cells and donor plasmid DNA at the two homologous parts of the gene, replacement of the chromosome sequence with the donor DNA occur and selective marker gene is inserted into the objective gene to be disrupted. In general, double crossing over type of plasmid has lower frequency of recombination than single crossing type of vector.

[0044] In this invention, inventors inactivated the enzymes of interest by their disruption of corresponding genes. The other way to evaluate an effect of gene product of an interest is to decrease its expression with genetic engineering methods. For this purpose, some methods were exploited. One of such methods is anti-sense method. Anti-sense method is a method to decrease an expression of gene of interest by introducing an artificial gene fragment, whose sequence is complementary to that of gene of interest. And such an anti-sense gene fragment form complex with mature mRNA fragment of objective gene *in vivo* and inhibit an efficient translation from mRNA, as a consequence.

[0045] The other method is a mutation of promoter region. In general, the gene consists of several parts which have different functions from each other. In eukaryotes, genes which encode corresponding protein are transcribed to pre-mature messenger RNA (pre-mRNA) differing from the genes for ribosomal RNA (rRNA), small nuclear RNA (snRNA) and transfer RNA (tRNA). Although RNA polymerase II (PolII) plays a central role in this transcription event, PolII can not solely start transcription without *cis* element covering an upstream region containing a promoter and an upstream activation sequence (UAS), and a *trans*-acting protein factor. At first, a transcription initiation complex which consists of several basic protein components recognize the promoter sequence in the 5'-adjacent region of the gene to be expressed. In this event, some additional participants are required in the case of the gene which is expressed under some specific regulation, such as a heat shock response, or adaptation to a nutrition starvation, and so on. In such a case, a UAS is required to exist in the 5'-untranslated upstream region around the promoter sequence, and some positive or negative regulator proteins recognize and bind to the UAS. The strength of the binding of transcription initiation complex to the promoter sequence is affected by such a binding of the *trans*-acting factor around the promoter, and this enables the regulation of the transcription activity.

[0046] After the activation of a transcription initiation complex by the phosphorylation, a transcription initiation complex initiates transcription from the transcription start site. Some parts of the transcription initiation complex are detached as an elongation complex from the promoter region to the 3' direction of the gene (this step is called as a promoter clearance event) and an elongation complex continues the transcription until it reaches to a termination sequence that is located in the 3'-adjacent downstream region of the gene.

[0047] To decrease an expression of gene of interest, mutation by conventional chemical mutagenesis or genetic site-directed mutagenesis in the promoter region of objective gene containing UAS sequence described above was often used. Mutant strain in which expression of enzyme of interest might decrease can be obtained by transforming host strain with a recombinant DNA having such a mutated promoter region. As described above, such an attempt to decrease the expression of gene is also employed as well as gene disruption for objective to determine an effect of gene product on phenomena in living organisms.

[0048] As a transformation method, LiCl method (Wery *et al.*, *Yeast*, 12 (7), 641-651, 1996) and electroporation method (Wery *et al.*, *Gene*, 184, 89-97, 1997) were applied to transform *P. rhodozyma* but the efficiency of transformation under these conditions seemed to be still low. In this invention, Biolistic transformation method (Johnston *et al.*, *Methods in Molecular Biology*, 53; 147-153, 1996) was applied for transformation of *P. rhodozyma*. Biolistic method is a simple and reliable protocol, in which donor DNA coated on gold or tungsten particle is shot into the living cells directly with high-pressured helium gas. This transformation protocol was successfully applied to *Cryptococcus neoformans* which belongs to basidiomycetous yeast as well as *P. rhodozyma* and was difficult to transform with conventional transformation method (Toffaletti, *et al.*, *J. Bacteriol.*, 175 (5), 1405-1411, 1993). In this invention, this biolistic method was successfully used to transform *P. rhodozyma* cells.

[0049] The event of gene disruption can be confirmed by enzymatic characterization directly and by genetic analysis with PCR or Southern blot hybridization by using the chromosome obtained from transformants thus obtained. In this invention, a direct confirmation of SOD disruption were performed by activity staining and characterization of catalase disruption were conducted by visual observation like catalase test which is often used in bacterial taxonomy.

[0050] Such a genetically engineered *P. rhodozyma* would be cultivated in an appropriate medium and evaluated in its productivity of astaxanthin.

[0051] The present invention is further illustrated with Examples described below by referring to the attached drawings.

Example

[0052] The following materials and methods were employed in the Example described below:

Strain**[0053]**

- 5 *P. rhodozyma* ATCC 96594 (re-deposited under the accession No. ATCC 74438 on April 8, 1998 pursuant to the Budapest Treaty)
- E. coli* DH5a: F⁻, ϕ 80d, *lacZ* Δ M15, Δ (*lacZYA-argF*)U169, *hsd* (*r_K⁻*, *m_K⁺*), *recA1*, *end A1*, *deoR*, *thi-1*, *supE44*, *gyrA96*, *relA1* (Toyobo, Osaka, Japan)
- 10 *E. coli* XL 1 -Blue MRF': Δ (*mcrA*)183, Δ (*mcrCB-hsdSMR-mrr*)173, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac* [F' *proAB*, *lac1* Δ M15, Tn10 (*tet^r*)] (Stratagene, La Jolla, USA)
- 15 *E. coli* SOLR: *e14'* (*mcrA*), Δ (*mcrCB-hsdSMR-mrr*)171, *sbcC*, *recB*, *recJ*, *umuC* :: Tn5(*kan^r*), *uvrC*, *lac*, *gyrA96*, *relA1*, *thi-1*, *endA1*, λ ^R, [F' *proAB*, *lac* Δ M15] Su⁻ (nonsuppressing) (Stratagene)
- E. coli* XL1/MRA (P2): Δ (*mcrA*)183, Δ (*mcrCB-hsdSMR-mrr*)173, *endA1*, *supE44*, *thi-1*, *gyrA96*, *relA1*, *lac* (P2 lysogen) (Stratagene)
- 20 *E. coli* TOP10: F⁻, *mcrA*, Δ (*mrr-hsdRMS-mcr*)BC, ϕ 80, Δ *lacZ* M15, Δ *lacX*74, *recA1*, *deoR*, *araD139*, (*ara-leu*)7697, *galU*, *galK*, *rpsL* (*Str^r*), *endA1*, *nupG* (Invitrogen, Carlsbad, USA)

Vector**[0054]**

- λ ZAPII (Stratagene)
- λ DASHII (Stratagene)
- 30 pBluescriptII SK+ (Stratagene)
- pCR2.1TOPO (Invitrogen)
- 35 pUC19 (Takara Shuzo, Otsu, Japan)

Media

- 40 **[0055]** *P. rhodozyma* strain is maintained routinely on agar plate of YPD medium (DIFCO, Detroit, USA). *E. coli* strain is maintained in LB medium (10 g Bacto-trypton, 5 g yeast extract (DIFCO) and 5 g NaCl per liter). NZY medium (5 g NaCl, 2 g MgSO₄·7H₂O, 5 g yeast extract (DIFCO), 10 g NZ amine type A (WAKO, Osaka, Japan) per liter is used for λ phage propagation in a soft agar (0.7 % agar (WAKO)). When an agar medium was prepared, 1.5 % of agar (WAKO) was supplemented.

[0056] Methods

- [0056]** A general method for techniques of molecular genetics was according to the method in Molecular cloning: a Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory Press, 1989). Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Japan).
- 50 **[0057]** Isolation of a chromosomal DNA from *P. rhodozyma* was performed by using QIAGEN Genomic Kit (QIAGEN, Hilden, Germany) following the protocol supplied by the manufacturer. Mini-prep of plasmid DNA from transformed *E. coli* was performed with the Automatic DNA isolation system (PI-50, Kurabo, Co. Ltd., Osaka, Japan). Midi-prep of plasmid DNA from an *E. coli* transformant was performed by using QIAGEN column (QIAGEN). Isolation of λ DNA was performed by Wizard lambda preps DNA purification system (Promega, Madison, USA) following the protocol prepared by the manufacturer. A DNA fragment was isolated and purified from agarose by using QIAquick or QIAEX II (QIAGEN).
- 55 **[0058]** Isolation of total RNA from *P. rhodozyma* was performed with phenol method by using Isogen (Nippon Gene, Toyama, Japan). mRNA was purified from total RNA thus obtained by using mRNA separation kit (Clontech, Palo Alto,

USA). cDNA was synthesized by using CapFinder cDNA construction kit (Clontech).

[0059] *In vitro* packaging was performed by using Gigapack III gold packaging extract (Stratagene).

[0060] Polymerase chain reaction (PCR) is performed with the thermal cycler from Perkin Elmer model 2400. Each PCR condition is described in examples. PCR primers were purchased from a commercial supplier or synthesized with a DNA synthesizer (model 392, Perkin Elmer, Japan, Urayasu, Japan). Fluorescent DNA primers for DNA sequencing were purchased from Pharmacia. DNA sequencing was performed with the automated fluorescent DNA sequencer (ALFred, Pharmacia).

[0061] Competent cells of *E. coli* DH5 α were purchased from Toyobo (Japan).

[0062] The apparatus and reagent for biolistic transformation of *P. rhodozyma* were purchased from Nippon Bio-Rad Laboratories (Tokyo, Japan).

Example 1: Isolation of mRNA from *P. rhodozyma* and construction of cDNA library

[0063] To construct cDNA library of *P. rhodozyma*, total RNA was isolated by phenol extraction method right after the cell disruption and the mRNA from *P. rhodozyma* ATCC96594 strain was purified by using mRNA separation kit (Clontech).

[0064] At first, Cells of ATCC96594 strain from 10 ml of two-day-culture in YPD medium were harvested by centrifugation (1500 x g for 10 min.) and washed once with extraction buffer (10 mM Na-citrate / HCl (pH 6.2) containing 0.7 M KCl). After suspending in 2.5 ml of extraction buffer, the cells were disrupted by French press homogenizer (Ohtake Works Corp., Tokyo, Japan) at 1500 kgf/cm² and immediately mixed with two times of volume of isogen (Nippon gene) according to the method specified by the manufacturer. In this step, 400 μ g of total RNA was recovered.

[0065] Then this total RNA was purified by using mRNA separation kit (Clontech) according to the method specified by the manufacturer. Finally, 16 μ g of mRNA from *P. rhodozyma* ATCC 96594 strain was obtained.

[0066] To obtain cDNA species from *P. rhodozyma*, CapFinder PCR cDNA construction kit (Clontech) was used according to the method specified by the manufacturer. One μ g of purified mRNA was applied for a first strand synthesis followed by PCR amplification. After this amplification by PCR, 1 mg of cDNA pool was obtained.

Example 2: Cloning of two species of partial SOD gene from *P. rhodozyma*

[0067] To clone a partial SOD gene from *P. rhodozyma*, a degenerate PCR method was exploited. Two mixed primers whose nucleotide sequences were designed and synthesized as shown in TABLE 1 based on the common sequence of known superoxide dismutase genes from other species.

TABLE 1

Sequence of primers used in the cloning of SOD1 and SOD2 genes

Sod1 ; AARCAYCAYCARACNTAYGTNAA (sense primer) (SEQ ID NO: 10)

Sod4 ; GCCCANCCNGANCCYTGNACNCC (antisense primer) (SEQ ID NO: 11)

(R=A or G; Y=C or T; N=A, C, G or T)

[0068] After the PCR reaction of 25 cycles of 94 °C for 15 seconds; 46 °C for 30 seconds and 72°C for 15 seconds by using ExTaq (Takara Shuzo) as a DNA polymerase and cDNA pool obtained in example 1 as a template, reaction mixture was applied to agarose gel electrophoresis. A PCR band that has a desired length was recovered and purified by QIAquick (QIAGEN) according to the method by the manufacturer and then ligated to pCR2.1-TOPO (Invitrogen). After the transformation of competent *E. coli* TOP10, 6 white colonies were selected and plasmids were isolated with Automatic DNA isolation system (Kurabo PI-50). As a result of sequencing, it was found that two clones had different sequences each other, whose deduced amino acid sequences were independently similar to known SOD genes. These isolated cDNA clones were designated as pSOD614 #2 and pSOD614 #3 and used for further study.

Example 3: Isolation of genomic DNA from *P. rhodozyma*

[0069] To isolate a genomic DNA from *P. rhodozyma*, QIAGEN genomic kit was used according to the method spec-

ified by the manufacturer.

[0070] At first, cells of *P. rhodozyma* ATCC 96594 strain from 100 ml of overnight culture in YPD medium were harvested by centrifugation (1500 x g for 10 min.) and washed once with TE buffer (10 mM Tris / HCl (pH 8.0) containing 1 mM EDTA). After suspending in 8 ml of Y1 buffer of the QIAGEN genomic kit, lyticase (SIGMA) was added at the concentration of 2 mg/ml to disrupt cells by enzymatic degradation and the reaction mixture was incubated for 90 minutes at 30 °C and then proceeded to the next extraction step. Finally, 20 µg of genomic DNA was obtained.

Example 4: Southern blot hybridization by using pSOD614 #2 and pSOD614 #3 as probes

[0071] Southern blot hybridization was performed to clone genomic fragment which contains *SOD* genes from *P. rhodozyma*. Two µg of genomic DNA was digested by *EcoRI* and subjected to agarose gel electrophoresis followed by acidic and alkaline treatment. The denatured DNA was transferred to nylon membrane (Hybond N+, Amersham) by using transblot (Joto Rika, Tokyo, Japan) for an hour. The DNA was transferred to nylon membrane and fixed by a heat treatment (80 °C, 90 minutes). Probes were prepared by labeling the template DNAs (*EcoRI*- digested pSOD614 #2 and pSOD614 #3) with DIG multipriming method (Boehringer Mannheim). Hybridization was performed with the method specified by the manufacturer. As a result, hybridized bands were visualized in the length of 7.5 kilobases (kb) against the probe prepared from pSOD614 #2, and in the length of 8.0 kilobases (kb) against the probe prepared from pSOD614 #3.

Example 5: Cloning of genomic fragments containing *SOD* genes whose sequences were different from each other

[0072] Four µg of the genomic DNA was digested by *EcoRI* and subjected to agarose gel electrophoresis. Then, DNAs whose length are within the range from 7 to 9 kb were recovered by a conventional elution method by using dialysis membrane. The purified DNA was ligated to 1 µg of *EcoRI*-digested and CIAP (calf intestine alkaline phosphatase) - treated λZAPII (Stratagene) at 16 °C overnight, and packaged with Gigapack III gold packaging extract (Stratagene). The packaged extract was infected to *E. coli* XL1Blue MRF' strain and over-laid with NZY medium poured onto LB agar medium. About 6000 plaques were screened by using *EcoRI*- digested pSOD614 #2 and pSOD614 #3 as probes. Six plaques were hybridized to the labeled pSOD614 #2 probe and two plaques were hybridized to the labeled pSOD614 #3 probe. Then, hybridized plaques were subjected to *in vivo* excision protocol according to the method specified by the manufacturer (Stratagene). It was found that one plasmid isolated from six of pSOD614 #2-hybridized plaques had the same fragments as that of pSOD614 #2 as a result of PCR analysis by using *sod1* and *sod4* primers. This plasmid was named as pSOD703. It was also found that two plasmids isolated from two of pSOD614 #3-hybridized plaques had the same fragment as that of pSOD614 #3 as a result of PCR analysis by using *sod1* and *sod4* primers. One of the plasmids was named as pSOD626 and used for further study.

Example 6: Sequence analysis of two species of MnSOD genes obtained from *P. rhodozyma*

[0073] A complete nucleotide sequence was determined by sequencing of pSOD703 and pSOD626 with a primer-walking procedure. Nucleotide and their deduced amino acid sequences for *SOD1* gene which harbored on pSOD703 and for *SOD2* gene which harbored on pSOD626 are listed as SEQ ID NO: 1, NO: 2, NO: 5 and NO: 7 in the sequence listing section.

[0074] Both of deduced amino acid sequences of *SOD1* and *SOD2* genes were homologous to known MnSODs obtained from other species and not to Cu/ZnSODs or FeSOD as a result of BLAST analysis (Altschul, S.F. *et al.*, *J. Mol. Biol.* 215, 403-410, 1990).

[0075] *SOD1* gene had 7 introns and 8 exons. And its deduced open reading frame consisted of 223 amino acids. On the other hand, *SOD2* gene had 10 introns and 11 exons, and its deduced open reading frame consisted of 199 amino acids. Most difference between two isolated *SOD* genes was extended region of *SOD1* gene in its amino terminal end, whose sequence might act as a transit peptide to mitochondria

[0076] In fact, Schroeder *et al.* reported two species of SODs which were detected as KCN and H₂O₂ resistant SODs in activity staining of native polyacrylamide gel electrophoresis (PAGE) in *P. rhodozyma*. They commented two species of MnSOD were indicated as aggregates or isozymes and did not refer to their precise nature and their subcellular location. As described in the following section, it was clarified that two species of those KCN- and H₂O₂- resistant SODs (i.e. MnSOD) were products of *SOD1* and *SOD2* genes. As described in the section of "detailed description of the invention", most eucaryotes have different species of SODs in their intracellular location (MnSOD in the mitochondrial fraction and Cu/ZnSOD in the cytoplasmic fraction). This is the first example in which two species of MnSODs functioned in a different subcellular location.

Example 7: Construction of disruption plasmids for *SOD1* and *SOD2* genes

[0077] As described in the section "detailed description of the invention", a plasmid harboring a drug resistant marker cassette was constructed by inserting G418 resistant structure gene between promoter and terminator genes of glyceraldehyde-3-phosphate dehydrogenase (GAP) and ligating into *KpnI*- and *HindIII*- digested pUC19. This plasmid was named as pUC-G418 and used for a further study. As gene fragments which were used for homologous recombination, partial fragments of *SOD1* and *SOD2* genes were synthesized *in vitro* by PCR method using PCR primers whose sequences were shown in TABLE 2.

TABLE 2

Sod14 ; GGTACCTCCGATGATAGGAATGTGAG (sense primer) (SEQ ID NO: 12)

Sod15 ; GAATTCAGTTCAACGGAGGAGGACAC (antisense primer) (SEQ ID NO: 13)

Sod47; GAATTCGGAGGAGGACACATCAACCG (sense primer) (SEQ ID NO: 14)

Sod48; GGTACCTGTACTGGAGGTAGAAAGCG (antisense primer) (SEQ ID NO: 15)

[0078] PCR condition was as follows; 25 cycles of 94 °C for 15 seconds, 50 °C for 30 seconds and 72 °C for 15 minutes. As a template, 0.1 µg of genomic DNA obtained in Example 3 was used, and ExTaq as a DNA polymerase. A partial fragment of *SOD1* which could be obtained from PCR using sod14 and sod15 as primers and a partial *SOD2* gene which could be obtained from PCR using sod47 and sod48 as primers were amplified respectively. Each amplified 0.65 kb fragments was recovered and cloned in pCR2.1-TOPO (Invitrogen) according to the protocol specified by the manufacturer. Six independent clones from white colonies of *E. coli* TOP10 transformants were selected and plasmids were prepared from those transformants. As a result of restriction analysis and sequencing, one clone which had a partial *SOD1* gene was selected for a further study and named as pSOD715. In a similar manner, one clone which had a partial *SOD2* gene was selected and named as pSOD826.

[0079] Then, pSOD715 and pSOD826 were digested by *EcoRI* and *KpnI*, and 0.65kb fragments yielded were purified by using QIAquick protocol and ligated to *EcoRI*- and *KpnI*- digested pUC19-G418. Six independent clones from ampicillin-resistant colonies of *E. coli* DH5α transformants were selected and plasmids were prepared from those transformants. As a result of restriction analysis and sequencing, one clone in which a partial *SOD1* was fused to G418 resistant cassette was obtained and named as pSOD/G717. In a similar manner, one clone in which a partial *SOD2* was fused to G418 resistant cassette was obtained and named as pSOD/G828.

Example 8: Transformation of *P. rhodozyma* ATCC96594 with biolistic method

[0080] Transformation protocol were followed to the method described in Methods in Molecular Biology (Johnston *et al.*, 53; 147-153, 1996). As a host strain, *P. rhodozyma* ATCC 96594 was cultured in YPD medium to stationary phase. After centrifugation of broth, cells were concentrated by 10-fold with sterilized water and 200 µl of cell suspension were spread on YPD medium containing 100 µg of geneticin, and 0.75 M of mannitol and sorbitol. Five micrograms of circular DNA of pSOD/G717 and pSOD/G828 were coated on 1.5 mg of 0.9 µm gold particle, and used as donor DNA for biolistic transformation. Hundreds of geneticin-resistant clones were yielded after one week of incubation at 20 °C. Four of those transformants were selected and chromosomes were prepared from them. One of the transformants was confirmed to have disrupted structure of chromosomal *SOD1* or *SOD2* gene by PCR and Southern blot hybridization analyses, and was used for further study.

Example 9: Activity staining of native PAGE by using crude extracts which obtained from candidates for *SOD1* and *SOD2* disruptants

[0081] ATCC96594 strain and candidates which obtained from biolistic transformation of ATCC96594 were cultivated in YPD medium for two days and harvested by centrifugation for 10 min. by 3000 x g at 4 °C. After wash with Tris-HCl buffer (10mM / pH 8.0), cells were concentrated by 10-fold with the same buffer. Cells were disrupted with French

press homogenizer (Ohtake Works) at 1500 kg/cm² and crude extract was prepared after a microcentrifugation at 15000 rpm (TOMY, MRX150) of homogenized fraction.

[0082] Protein concentration of crude extract thus prepared was determined with BCA protein assay reagent released by PIERCE (Rockford, U.S.A.). A volume of crude extract corresponding to 300 µg of protein were subjected to native PAGE according to the method described by Schroeder W. A. *et al.* (*J. Gen. Microbiol.*, 139, 907-912, 1993). Method of activity staining was referred to the method by Flohé *et al.* (*Methods in Enzymology*, 105, 93-104, 1984).

[0083] Result of activity staining is depicted in FIG. 1. In the extract of parental strain, ATCC96594, two bands were visualized as transparent bands which have SOD activity in the dark background. On the contrary, ATCC96594 :: pSOD/G717 strain in which *SOD1* gene was disrupted, lacked activity band with high mobility in native PAGE and ATCC96594 :: pSOD/G828 strain, in which *SOD2* gene was disrupted lacks activity band with low mobility in native PAGE. From this result, it was found that two species of MnSOD that were present in the crude extract of *P. rhodozyma* were products of *SOD1* and *SOD2* genes, and SOD species with high mobility and with low mobility in native PAGE corresponded to *SOD1* and *SOD2* gene product, respectively.

Example 10: Cloning of partial catalase (*CAT*) gene from *P. rhodozyma*

[0084] To clone a partial *CAT* gene from *P. rhodozyma*, a degenerate PCR method was exploited. Two mixed primers whose nucleotide sequences were designed and synthesized as shown in TABLE 3 based on the common sequence of known catalase genes from other species.

TABLE 3

Sequence of primers used in the cloning of *CAT* genes

Cat2 ; NIGNTTYTCNACNGTNGGNGGNGA (sense primer) (SEQ ID NO: 16)

Cat5 ; CKRTGNCKYTGNGTRTCNGGRTA (antisense primer) (SEQ ID NO: 17)
(M=A or C; N=A, C, G or T; Y=C or T; K=G or T; R=A or G)

[0085] After the PCR reaction of 25 cycles of 94 °C for 15 seconds, 45 °C for 30 seconds and 72°C for 15 seconds by using ExTaq (Takara Shuzo) as a DNA polymerase and genomic DNA obtained in example 3 as a template, reaction mixture was applied to agarose gel electrophoresis. A PCR band that has a 1.0 kb length was recovered and purified by QIAquick (QIAGEN) according to the method by the manufacturer and then ligated to pCR2.1-TOPO (Invitrogen). After the transformation of competent *E. coli* TOP 10, 6 white colonies were selected and plasmids were isolated with Automatic DNA isolation system. As a result of sequencing, it was found that two clones had sequence whose deduced amino acid sequences was similar to known *CAT* genes. One of these isolated DNA clones was designated as pCAT702 and used for further study.

Example 1 Cloning of genomic fragments containing *CAT* gene

[0086] In a similar manner to Example 4, Southern blot hybridization study was performed by using pCAT702 as a probe. As a result, hybridized band which had a size from 9 kb to 23 kb was visualized. Next, 4 µg of the genomic DNA was digested by *EcoRI* and subjected to agarose gel electrophoresis. Then, DNAs whose length is within the range from 9 to 23 kb was recovered by a conventional elution method by using dialysis membrane. The purified DNA was ligated to 1 µg of *EcoRI*-digested and CIAP (calf intestine alkaline phosphatase) - treated λDASHII (Stratagene) at 16 °C overnight, and packaged by Gigapack III gold packaging extract (Stratagene). The packaged extract was infected to *E. coli* XL1Blue MRA(P2) strain and over-laid with NZY medium poured onto LB agar medium. About 8000 plaques were screened using *EcoRI*- digested pCAT702 as a probe. Six plaques were hybridized to the labeled pCAT702 probe. λDNA was prepared from each λ clone and it was found that 4 of 6 clones contained same fragment to the insert of pCAT702 as a result of PCR using Cat2 and Cat5 primers, and sequencing analysis. A partial nucleotide and its deduced amino acid sequence for *CAT* gene are listed as SEQID NO: 3 and SEQID NO: 9 in the sequence listing section.

Example 12 Construction of disruption plasmid for *CAT* gene

[0087] In a similar manner to Example 7, disruption plasmid for *CAT* gene was constructed. At first, *SacI* linker was

inserted at *Hind*III site of pUC19-G418, in which terminator region of G418-resistant cassette was located, and as a result of restriction analysis, pUC19-G418Sa, which had *Sac*I site at the end of the G418-resistant cassette was yielded. Then, *Kpn*I- and *Sac*I- fragment derived from pUC19-G419Sa was ligated to *Kpn*I- and *Sac*I- digested pCAT702 and yielded pCAT/G706, in which a partial genomic *CAT* gene was fused to G418-resistant cassette.

Example 13 Transformation of *P. rhodozyma* ATCC 96594 by using pCAT/G706 as donor plasmid

[0088] In a same manner to Example 8, *P. rhodozyma* ATCC 96594 was transformed with *CAT*-disruption plasmid, pCAT/G706. Hundreds of geneticin-resistant clones were yielded after one week of incubation at 20 °C. Four of those transformants were selected and chromosomes were prepared from them. One of the transformants was confirmed to have disrupted structure of chromosomal *CAT* gene by PCR and Southern blot hybridization analyses, and was used for further study.

[0089] Then, two candidates for *CAT* disruptant were characterized with catalase test, which was often used in bacterial taxonomic study. One loopful of cells of *P. rhodozyma* was soaked in 3% H₂O₂ solution and then occurrence of dioxygen gas was observed. Although an immediate occurrence of O₂ foam were confirmed when ATCC 96594 cells were applied to this catalase test, O₂ foam was occurred after a long lag when two ATCC 96594 :: pCAT/G706 mutants were soaked in H₂O₂ solution. From this result, disruption of *CAT* gene was suggested, but a remaining weak activity indicated a presence of another player who catalyzes disappearance of H₂O₂ such as peroxidase in *P. rhodozyma*.

Example 14 Evaluation of *SOD1*, *SOD2* and *CAT* disruptants derived from *P. rhodozyma* for their astaxanthin production

[0090] Effect of gene disruption of *SOD1*, *SOD2* and *CAT* gene on astaxanthin production was evaluated by cultivation in YPD medium with shaking flasks. Cells which grew on YPD agar were suspended in YPD medium and a portion of cell suspension was inoculated to 50 ml of YPD medium in 500 ml baffled flask. Cells were grown with 200 r. p. m. at 20 °C for 84 hours. At an appropriate interval, 3 ml of broth was withdrawn and was analyzed for cell yield, consumption of glucose and astaxanthin content.

[0091] Cell yield was measured as optical density at 660 nm and as dry cell weight by weighing cells after filtration through 0.45 µm cellulose acetate plus nitrocellulose membrane (HAWP04700, Millipore, Bedford, U.S.A.) and heating at 80 °C overnight. Astaxanthin content of *P. rhodozyma* was measured with HPLC method after extraction of carotenoids from cells of *P. rhodozyma* by disruption with glass beads. After extraction, 5ml of acetone/BHT/water containing appropriate concentration of bixin as an internal standard was added. Supernatant was analyzed for astaxanthin content with following HPLC system.

HPLC column; YMC-Pak ODS-A (6 mm, 150 mm)

Temperature; room temperature

Eluent; acetonitrile / methanol / isopropanol (85/10/5)

Injection volume; 10 µl

Flow Rate; 2.0 ml/minute

Detection; UV at 471 nm

Results obtained from 84 hour-culture are summarized in TABLE 4.

TABLE 4

The effect of <i>SOD</i> and <i>CAT</i> mutation on productivity of total carotenoids and astaxanthin by <i>P. rhodozyma</i>		
Strain	Total carotenoids (mg/g-dry-cell)	astaxanthin (mg/g-dry cell)
ATCC 96594	0.169	0.111
Δ <i>SOD1</i>	0.259	0.146
Δ <i>SOD2</i>	0.202	0.129
Δ <i>CAT</i>	0.229	0.144

[0092] *SOD1* and *SOD2* disruptants showed elevated level of productivity for total carotenoids as well as astaxanthin compared to their host strain, ATCC 96594. Especially, *SOD1* disruptant showed significant increase of carotenoids and astaxanthin production by 53.3 % and 31.5 %, respectively.

[0093] *SOD1* seemed to be a mitochondrial enzyme judged from deduced transit peptide sequence at its amino terminal end and might act to scavenge superoxide radical, a kind of active oxygen species occurred in the respiratory chain at mitochondria. These data suggested that astaxanthin production was stimulated by a generation of intracellular active oxygen to compensate the lack of native player of active oxygen species-quenching factor, *SOD1*.

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		165	170	175
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Gln Val Gln Val Met Ser Pro Glu Gln Ala Gln Lys Phe Arg Tyr Asn
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55 Ile Leu Asp Leu Thr Lys Val Trp Ser His Lys Glu Phe Pro Leu Arg
180 185 190

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Thr Ile Gly Lys Phe Thr Leu Asn Arg Asn Val Asp Asn Tyr Phe Ala
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Glu Pro Ser Asn Asp Pro Val Leu Gln Ala Arg Leu Phe Ser
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23

Claims

1. A process for producing carotenoids which comprises cultivating a recombinant organism whose gene for one or more active oxygen species-quenching factor(s) is substantially disrupted with the aid of a disruption cassette specific to the said gene, and recovering carotenoids from the culture.
2. A process according to claim 1, wherein the host organism of the said recombinant organism belongs to the kingdom of *Monera*, *Protista* or *Fungi*.

3. A process according to claim 1 or 2, wherein the host organism of the said recombinant organism belongs to the genus *Erwinia*, *Rhodobacter*, *Myxococcus*, *Flavobacter*, *Paracoccus*, *Synechococcus*, *Synechocystis*, *Agrobacterium*, *Streptomyces*, *Haematococcus*, *Dunaliella*, *Phaffia*, *Xanthophyllomyces*, *Neurospora*, *Rhodotorula*, *Blakeslea*, or *Phycomyces*.
- 5 4. A process according to claim 3, wherein the host organism of the said recombinant organism is a strain of *P. rhodozyma*.
- 10 5. A process according to any one of claims 1 - 4, wherein the active oxygen species-quenching factor(s) is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD) and/or catalase.
- 15 6. A recombinant organism producible of carotenoids, which is characterized in that the gene for at least one active oxygen species-quenching factor is substantially disrupted with the aid of introduction of a disruption cassette specific to the said gene.
- 20 7. A recombinant organism according to claim 6, wherein the host organism of the said recombinant organism belongs to the kingdom of *Monera*, *Protista* or *Fungi*, more preferably to the genus *Erwinia*, *Rhodobacter*, *Myxococcus*, *Flavobacter*, *Paracoccus*, *Synechococcus*, *Synechocystis*, *Agrobacterium*, *Streptomyces*, *Haematococcus*, *Dunaliella*, *Phaffia*, *Xanthophyllomyces*, *Neurospora*, *Rhodotorula*, *Blakeslea*, or *Phycomyces*.
- 25 8. A recombinant organism according to claim 6 or 7, wherein the active oxygen species-quenching factor(s) to be disrupted is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD) and/or catalase.
- 30 9. A disruption cassette for disrupting a gene coding for an active oxygen species-quenching factor effective on carotenogenesis in an organism which is producible of carotenoids, which comprises a partial nucleotide sequence substantially identical to a part of the DNA sequence coding for an active oxygen species-quenching factor and a selectable marker gene.
- 35 10. A disruption cassette according to claim 9, wherein the said organism belongs to the kingdom of *Monera*, *Protista* or *Fungi*, more preferably to the genus *Erwinia*, *Rhodobacter*, *Myxococcus*, *Flavobacter*, *Paracoccus*, *Synechococcus*, *Synechocystis*, *Agrobacterium*, *Streptomyces*, *Haematococcus*, *Dunaliella*, *Phaffia*, *Xanthophyllomyces*, *Neurospora*, *Rhodotorula*, *Blakeslea*, or *Phycomyces*.
- 40 11. A disruption cassette according to claim 9 or 10, wherein the active oxygen species-quenching factor(s) to be disrupted is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD) and/or catalase.
- 45 12. A disruption cassette according to any one of claims 9 to 11, wherein the partial nucleotide sequence coding for an active oxygen species-quenching factor is identical to at least a part of the DNA sequence coding for the active oxygen species-quenching factor of the organism into which the disruption cassette is to be introduced.
13. A disruption cassette according to claim 12, wherein the partial nucleotide sequence substantially identical to a part of the DNA sequence coding for an active oxygen species-quenching factor comprises at least one deletion and/or mutation compared to the corresponding functional gene.
14. A recombinant DNA sequence coding for an active oxygen species-quenching factor effective on carotenogenesis in an organism producible of carotenoids.
- 50 15. A recombinant DNA sequence according to claim 14, wherein the said organism belongs to the kingdom of *Monera*, *Protista* or *Fungi*.
- 55 16. A recombinant DNA sequence according to claim 14 or 15, wherein the said organism belongs to the kingdom of *Monera*, *Protista* or *Fungi*, more preferably to the genus *Erwinia*, *Rhodobacter*, *Myxococcus*, *Flavobacter*, *Paracoccus*, *Synechococcus*, *Synechocystis*, *Agrobacterium*, *Streptomyces*, *Haematococcus*, *Dunaliella*, *Phaffia*, *Xanthophyllomyces*, *Neurospora*, *Rhodotorula*, *Blakeslea*, or *Phycomyces*.
17. A recombinant DNA sequence according to any one of claims 14 - 16, which is a sequence originated from a

microorganism of *P. rhodozyma*.

18. A recombinant DNA sequence according to any one of claims 14 - 17, wherein the active oxygen species-quenching factor is mitochondrial superoxide dismutase.
19. A recombinant DNA sequence according to claim 18, wherein the sequence is identified by SEQ ID NO: 1 or 4, or has high homology to the sequence of SEQ ID NO: 1 or 4 enough to recombine with either of the sequences SEQ ID NOs: 1 and 4.
20. A recombinant DNA sequence according to claim 14 - 17, wherein the active oxygen species-quenching factor is cytoplasmic superoxide dismutase.
21. A recombinant DNA sequence according to claim 20, wherein the sequence is identified by SEQ ID NO: 2 or 6, or has high homology to the sequence of SEQ ID NO: 2 or 6 enough to recombine with either of the sequences of SEQ ID NOs: 2 and 6.
22. A recombinant DNA sequence according to claim 14 - 17, wherein the active oxygen species-quenching factor is catalase.
23. A recombinant DNA sequence according to claim 22, wherein the sequence is identified by SEQ ID NO: 3 or 8, or has high homology to the sequence of SEQ ID NO: 3 or 8 enough to recombine with either of the sequences of SEQ ID NOs: 3 and 8.
24. A recombinant DNA fragment which comprises a coding region for a transit peptide upstream the coding region of an objective protein.
25. A recombinant DNA fragment according to claim 24, wherein the objective protein is mitochondrial superoxide dismutase.
26. A method for locating an objective protein in mitochondria which comprises expressing the recombinant DNA fragment defined in claim 24 or 25 in an appropriate recombinant host organism.
27. A polynucleotide for use as probe or primer for cloning gene for the active oxygen species-quenching factor(s) effective on carotenogenesis in an organism producible of carotenoids.
28. A polynucleotide according to claim 27, wherein the active oxygen species-quenching factor(s) is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD) and/or catalase.

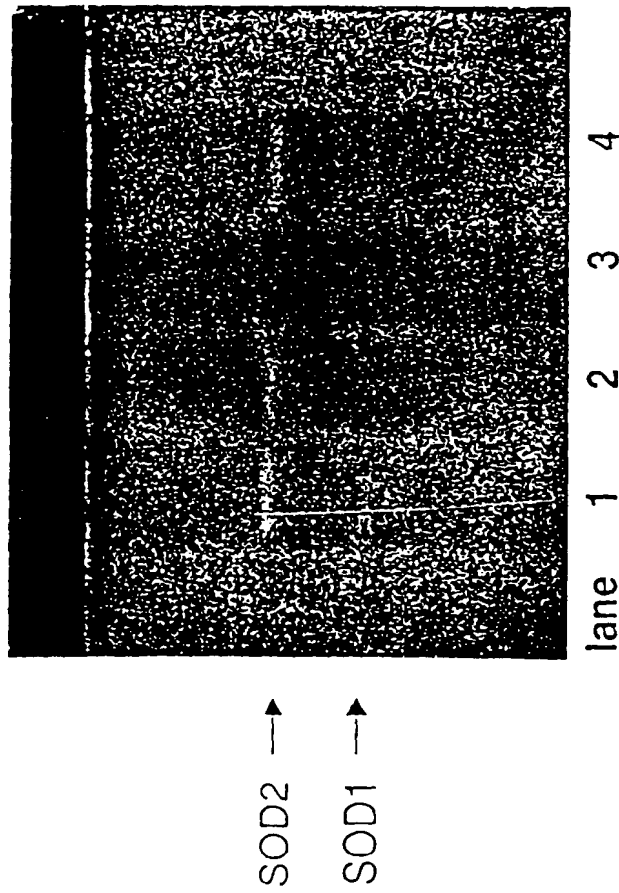
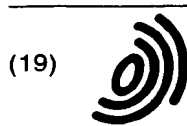


FIG. 1 Activity staining for super oxide dismutase
in SOD disruptants and their host strain, ATCC96594

lane 1 & 4 ; ATCC96594

lane 2 ; ATCC96594 :: pSOD/G717 (Δ SOD1)

lane 3 ; ATCC96594 :: pSOD/G828 (Δ SOD2)



(19)

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European Patent Office
Office européen des brevets



(11)

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(12)

EUROPEAN PATENT APPLICATION

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C12N 9/02

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4070 Basel (CH)

(54) Recombinant production of carotenoids, particularly astaxanthin

(57) The present invention is directed to a process for producing carotenoids comprising cultivating a recombinant organism having a gene for one or more active oxygen species-quenching factor(s) that is substantially disrupted with a disruption cassette specific to the

gene, and recovering carotenoids from the culture, as well as to genetic materials useful in the said process, such as a recombinant organism producible of carotenoids, a disruption cassette, a recombinant DNA sequence, a recombinant DNA fragment; and a polynucleotide.

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Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 12 6114

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
D,A	SCHROEDER WILLIAM A ET AL: "Antioxidant role of carotenoids in Phaffia rhodozyma." JOURNAL OF GENERAL MICROBIOLOGY, vol. 139, no. 5, 1993, pages 907-912, XP000993067 ISSN: 0022-1287 * the whole document *	1-13	C12P23/00 C12N9/08 C12N9/02
A	AN GIL-HWAN: "Photosensitization of the yeast Phaffia rhodozyma at a low temperature for screening carotenoid hyperproducing mutants." APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 66, no. 3, 1997, pages 263-268, XP000993071 ISSN: 0273-2289 * the whole document *	1-13	
D,A	SHAISH AVIV ET AL: "Are active oxygen species involved in induction of beta-carotene in Dunaliella bardawil?" PLANTA (HEIDELBERG), vol. 190, no. 3, 1993, pages 363-368, XP000993142 ISSN: 0032-0935 * the whole document *	1-13	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12P C12N
A	SCHROEDER WA ET AL.: "Singlet oxygen and peroxy radicals regulate carotenoid biosynthesis in Phaffia rhodozyma." J BIOL CHEM 1995 AUG 4;270(31):18374-9., XP000577110 * the whole document *	1-13	
-/--			
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 14 August 2001	Examiner Andres, S
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application I : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03/82 (P04C01)



European Patent
Office

Application Number

EP 00 12 6114

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):
- ☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

- ☒ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.
- ☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:
- ☐ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 12 6114

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	MARTINEZ C ET AL.: "Genetic transformation of astaxanthin mutants of <i>Phaffia rhodozyma</i> ." ANTONIE VAN LEEUWENHOEK 1998 FEB:73(2):147-53., XP000993172 ----		
X	CORTEZ NESTOR ET AL: "Molecular cloning and expression analysis of the <i>Rhodobacter capsulatus</i> <i>sodB</i> gene, encoding an iron superoxide dismutase." JOURNAL OF BACTERIOLOGY, vol. 180, no. 20, October 1998 (1998-10), pages 5413-5420, XP002166756 ISSN: 0021-9193 * the whole document *	6-16,20, 27,28	
X	JAKOPIITSCH CHRISTA ET AL: "Catalase-peroxidase from the cyanobacterium <i>Synechocystis</i> PCC 6803: Cloning, overexpression in <i>Escherichia coli</i> , and kinetic characterization." BIOLOGICAL CHEMISTRY, vol. 380, no. 9, September 1999 (1999-09), pages 1087-1096, XP000993333 ISSN: 1431-6730 * the whole document *	14-16, 22,27,28	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
X	FORKL HUBERT ET AL: "Molecular cloning, sequence analysis and expression of the gene for catalase-peroxidase (<i>cpeA</i>) from the photosynthetic bacterium <i>Rhodobacter capsulatus</i> B10." EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 214, no. 1, 1993, pages 251-258, XP000993284 ISSN: 0014-2956 * the whole document *	14-16, 22,27,28	
		-/--	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 14 August 2001	Examiner Andres, S
CATEGORY OF CITED DOCUMENTS X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons & member of the same patent family, corresponding document			

EPC FORM 1503 (03.92) (F04C01)



European Patent
Office

**LACK OF UNITY OF INVENTION
SHEET B**

Application Number

EP 00 12 6114

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-23, 27-28

1.1. Claims: 1-13

A process for producing carotenoids in an organism by disrupting a gene encoding an oxygen species quenching factor, and the disruption cassette used.

1.2. Claims: 14-17, 27-28 (all partially) and claims 18-19

A recombinant DNA coding for a mitochondrial SOD of a carotenoid producing organism.

1.3. Claims: 14-17, 27-28 (all partially) and claims 20-21

A recombinant DNA coding for a cytoplasmic SOD of a carotenoid producing organism.

1.4. Claims: 14-17, 27-28 (all partially) and claims 22-23

A recombinant DNA coding for a catalase of a carotenoid producing organism.

2. Claims: 24-26

A recombinant DNA comprising a coding region for a transit peptide upstream of the coding region for a protein, and its use for locating the protein in mitochondria.

Please note that all inventions mentioned under item 1, although not necessarily linked by a common inventive concept, could be searched without effort justifying an additional fee.



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 12 6114

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	CHUNG H -J ET AL: "Duplicate genes for Fe-containing superoxide dismutase in Streptomyces coelicolor A3(2)" GENE,NL,ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 231, no. 1-2, 29 April 1999 (1999-04-29), pages 87-93, XP004166781 ISSN: 0378-1119 * the whole document *	6-16,20, 27,28	
X	WO 90 02804 A (PLANT GENETIC SYSTEMS NV) 22 March 1990 (1990-03-22) * abstract * * page 7, line 19 - page 8, line 17 * * page 13, line 9 - page 14, line 22 * * claims *	24-26	
X	CREISSEN GARY ET AL: "Simultaneous targeting of pea glutathione reductase and of a bacterial fusion protein to chloroplasts and mitochondria in transgenic tobacco." PLANT JOURNAL, vol. 8, no. 2, 1995, pages 167-175, XP002174824 ISSN: 0960-7412 * pGR40 *	24-26	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 14 August 2001	Examiner Andres, S
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons K : member of the same patent family, corresponding document	

EPO FORM 1533 03 82 (P04C01)



European Patent
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EUROPEAN SEARCH REPORT

Application Number
EP 00 12 6114

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	LIN CHI-TSAI ET AL: "Cloning and characterization of a cDNA for manganese superoxide dismutase from callus of sweet potato." JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY, vol. 45, no. 2, 1997, pages 521-525, XP002174825 ISSN: 0021-8561 * the whole document *	24,25	
X	ZHANG C ET AL: "PHOTOSENSITISATION PROPERTIES OF MITOCHONDRIALLY LOCALISED GREEN FLUORESCENT PROTEIN" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 242, no. 2, 14 January 1998 (1998-01-14), pages 390-395, XP001002769 ISSN: 0006-291X * the whole document *	24,26	
A	NEUPERT WALTER: "Protein import into mitochondria." ANNUAL REVIEW OF BIOCHEMISTRY, vol. 66, 1997, pages 863-917, XP001015498 ISBN: 0-8243-0866-2 * the whole document *	24-26	
P,X	WO 00 28041 A (MICROBIOLOGICAL RES AUTHORITY ;HALLIS BASSAM (GB); SILMAN NIGEL (G) 18 May 2000 (2000-05-18) * abstract * * page 5, line 10 - line 18 * * page 9, line 28 - page 11, line 6 * * claims; examples 1,2,7,11 *	24-26	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 14 August 2001	Examiner Andres, S
CATEGORY OF CITED DOCUMENTS X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document		T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date O: document cited in the application I: document cited for other reasons S: member of the same patent family, corresponding document	

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